OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 3
2021

BIOLOGICAL SCIENCES

OPEN ACCESS

ANTIBACTERIAL SUSCEPTABILITY OF *E. COLI* STRAINS ISOLATED FROM RAW MILK

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DOI: 10/38045/ohrm.2021.3.08 CZU: 637.12:579.842.11+615.33.015.8

Keywords: *Escherichia coli, strains, microorganisms, raw milk, antibiotic resistance, susceptibility.*

Introduction. *The processing of most raw milk products can lead to contamination with unwanted microflora due to poor sanitation and hygienic conditions. The inadequate antibiotic use over the past decades has led to the emergence and wide spread of bacterial populations, particularly of Escherichia coli, which developed resistance to antibacterial drugs.* ***Material and methods****. Raw milk samples were obtained from clinically healthy cows on farms from Kiev and Poltava regions to identify E. coli, Staphylococcus spp., Enterococcus spp. isolates. Antimicrobial susceptibility testing was performed using the EUCAST disk diffusion method and MU on "Determination of microbial susceptibility to antibacterial drugs".* ***Results****. The examined milk samples revealed the presence of E. coli, Staphylococcus spp. and Enterococcus spp. isolates, which proves poor sanitary and hygienic conditions of milk production process. Escherichia coli isolates were found susceptible to Ampicillin/sulbactam, Cefoxitin (100%), Meropenem, Tobramycin (100%), Netilin, Tigecycline, Nitroxoline (100%), Gatifloxacin, and Nitrofurantoin (100%). The studied E. coli isolates were found resistant to Ampicillin (100%), Imipenem, Tetracycline, and Doxycycline (100%). 41.7% of isolates of Staphylococcus epidermidis, Staphylococcus aureus were found resistant to Oxacillin, of which 90% were resistant to Benzylpenicillin and 20% to Rifampicin.* ***Conclusions.*** *The circulation of antibiotic-resistant Enterobacteriaceae strains among farm animals is a major problem requiring a strategy development aimed to prevent the emergence and spread of antibiotic resistance worldwide.*

Cuvinte cheie: *Escherichia coli, tulpini, microorganisme, lapte crud, rezistență la antibiotice, sensi-bilitate.*

SENSIBILITATEA LA ANTIMICROBIENE A TULPINILOR DE *E. COLI* ISOLATE DIN LAPTE CRUD

Introducere. *Prelucrarea produselor din lapte crud poate duce la contaminarea cu microfloră nedorită din cauza condițiilor sanitaro-igienice precare. Utilizarea inadecvată a antibioticelor în ultimele decenii a dus la apariția și răspândirea pe scară largă a populațiilor bacteriene, în special a Escherichia coli, care a dezvoltat rezistență la preparatele antibacteriene.* ***Material și metode.*** *Probele de lapte crud au fost obținute de la vaci sănătoase, din punct de vedere clinic, de la fermele din regiunile Kiev și Poltava, din care au fost isolate și identificate E. coli, Staphylococcus spp., Enterococcus spp. Testarea sensibilității la antimicrobiene a fost efectuată utilizând metoda disc difuziometrică recomandată de EUCAST și MU privind „Determinarea sensibilității microbiene la preparatele antibacteriene".* ***Rezultate.*** *Probele de lapte examinate au relevat prezența izolatelor de E. coli, Staphylococcus spp. și Enterococcus spp., ceea ce elucidează condițiile sanitaro-igienice precare ale procesului de producție a laptelui. Izolatele de E. coli au fost sensibile la Ampicilin/sulbactam, Cefoxitin (100%), Meropenem, Tobramicin (100%), Netilin, Tigeciclin, Nitroxolin (100%), Gatifloxacin și Nitrofurantoin (100%). Izolatele studiate de E. coli au prezentat rezistență la Ampicilin (100%), Imipenem, Tetraciclin și Doxiciclin (100%). 41,7% din izolatele de Staphylococcus epidermidis, Staphylococcus aureus au fost rezistente la Oxacilin, dintre care 90% au fost rezistente la Benzilpenicilin și 20% la Rifampicin.* ***Concluzii.*** *Circulația tulpinilor de Enterobacteriaceae rezistente la antibiotice printre animalele de fermă prezintă o problemă majoră care necesită dezvoltarea strategiei menită să prevină apariția și răspândirea rezistenței la antibiotice în întreaga lume.*

INTRODUCTION

The production of high-quality raw milk depends on various factors related to both genetics and physiological condition of the dairy cattle, as well as on the product manufacturing technology. Moreover, individual factors might have a remote impact on milk quality and safety. Thus, the use of antibiotics for therapeutic purposes in lactating animals can significantly affect the antibiotic-resistant properties of microorganisms found in milk and serve as one of the pathways for the spread of antibiotic-resistance genes in the environment (1). Furthermore, the antibiotic resistance of the same microbial strains, isolated from animals kept in the same room, may differ depending on the type of antibiotics used to treat cows at different stages of production. A research on raw drinking milk on retail sale in England revealed pathogenic agents or signs of poor zoosanitary guidelines in almost half of the samples studied (2). This problem occurs regard-less of the level of livestock farming and dairy industry development (3). More than 150 antibiotics are used in the production of livestock products used for human consumption and 90% of them are natural products of bacteria, fungi and semi-synthetic substances obtained as a result of natural products modification or even synthesis (4). The most widely used antimicrobial agents used in treatment of productive animals are β-lactams, tetracyclines, aminoglycosides, lincosa-mides, macrolides, and sulfonamides (5, 6). Close to ANVISA (7). Microorganisms isolated from lactating cows show resistance to both natural and synthetic antibiotics. *Escherichia coli* isolated from cattle rectum exhibited high resistance to ampicillin (59.09%) and tetracycline (43.43%) (8). Special attention should be paid to the commensal microbiota (*Escherichia coli*, *enterococci*). These bacteria can also acquire antimicrobial resistance due to the selective pressure and may act as reservoirs for antimicrobial resistance and virulence genes within the environment, as well as in food and agricultural animals, which are likely to transmit resistance to pathogenic bacteria (9). Previous researches suggest that *E. coli* may generally enhance the mutation rates of target cells contributing to antibiotic resistance (10). *Staphylococci* were found the most common pathogens isolated from milk samples taken from cows with clinical and subclinical mastitis across several countries. *Staphylococcus aureus* is the main pathogen of this genus, being responsible for up to 40% of all mastitis cases in some geographic regions (11). A thorough under-standing on antibiotic resistance mechanisms is paramount to developing new strategies for preventing the emergence of resistance (12).

MATERIAL AND METHODS

Milk samples (32) were obtained from clinically healthy cows from the farms of the Kiev and Poltava regions. Culture media were prepared and controlled according to ISO 11133:2014 Microbiology of food, animal feeding stuffs and water. Preparation, production, storage and performance testing of culture media. The nutrient media, commercial tests, and discs with antimicrobial drugs manufactured by HiMedia were used within the study. Isolation and identification of *E. coli* used the appropriate *ISO* 16649-2:2014 (*ISO* 16649-2:2001, ITD) Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of -glucuronidase-positive *Escherichia coli*. Part 2. Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl-*D*-glucuronide. Isolation and identification of *Staphylococcus spp.* was carried out in accordance with ISO 6888-1: 1999 / Amd 1: 2003. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) - Part 1: Technique using Baird-Parker agar medium - Amendment 1: Inclusion of precision data. Isolation and identification of *Enterococcus spp.* was carried out in accordance with SSU 8534: 2015 Food products. Method for detection and determination of Enterococci (8534: 2015 Food products Method for detection and enumeration of Enterococci). Antimicrobial susceptibility testing was performed using the EUCAST disk diffusion method and MU on "Determination of microorganisms susceptibility to antibacterial drugs" (MHU 2009) (13, 14). The study results were recorded and interpreted via an Automatic Colony Counters Scan® 500 manufactured by INTERSCIENCE.

RESULTS

The present study examined milk samples obtained from clinically healthy cows from livestock complexes located in the Kiev and Poltava regions. The results of bacteriological studies showed that *E. coli* and *Enterococcus spp. s*trains were found in 100% of raw milk samples; thus,

Staphylococcus spp. isolates – in 100%, including 87.5% of *Staphylococcus epidermis* and 12.5% of coagulase-positive *Staphylococcus aureus*. It should be noted that among *Staphylococcus* strains, 41.7% of isolates were resistant to Oxacillin, of which 90% were resistant to Benzylpenicillin and 20% to Rifampicin (fig. 1).

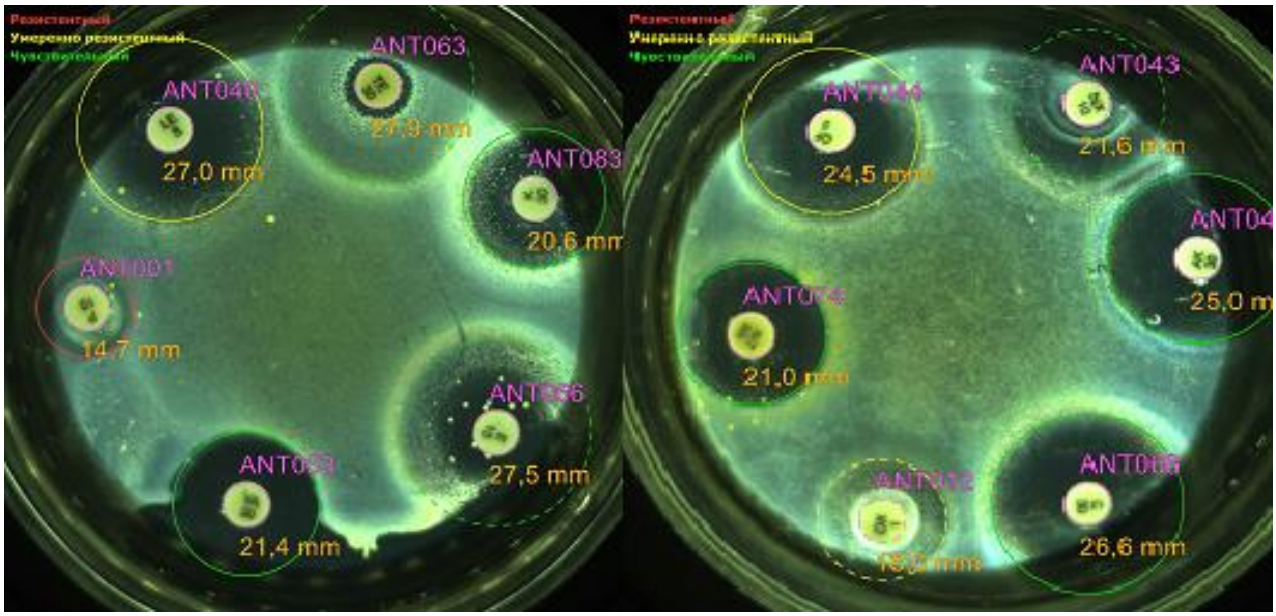

Figure 1. Antibiotic susceptibility of *Staphylococcus aureus* strains isolated from raw milk. ANT001 Benzylpenicillin (r), ANT012 Oxacillinum (r), ANT040 Levofloxacin (ATU), ANT043 Norfloxacin (s), ANT044 ofloxacin (ATU), ANT045 Amikacin (s), ANT053 Vancomycin (s), ANT056 Erythromycin (r), ANT063 Tetracyclinum (r), ANT068 Chloramphenicol (s), ANT074 Nitrofurantoin (s), ANT083 Kanamycin (r); «s» – sensitive; «r» – resistant; « ATU » – Area of Technical Uncertainty.

Antibiotic sensitivity in 24 *E. coli* strains isolated from raw milk was studied to beta-lactams from the groups of penicillins (semi-synthetic and inhibitor-protected drugs), cephalosporins (I-IV generations), carbarpenems; as well as the *E. coli* sensitivity to aminoglycosides (I-III generations), tetracyclines, quinolones (I-IV generations), Nitrofurantoin and Chloramphenicol.

E. coli sensitivity to the group of semi-synthetic penicillins, namely to Ampicillin, Piperacillin, Ticarcillin, Ampicillin/sulbactam, Ticarcillin/clavulanic acid was also studied. The research results showed that 100% of the studied cultures showed resistance to Ampicillin (fig. 2). 57.1% of strains were resistant to Piperacillin, 14.3% were moderately resistant, and 28.6% of the studied cultures were sensitive. The studied cultures exhibited resistance to Ticarcillin in 50%, 14.3% and 35.7% respectively.

Most of the studied isolates showed sensitivity to Ampicillin/sulbactam viz. in 95.8% of cases, thus showing resistance in 4.2% of *E. coli* isolates. 75% of isolates showed resistance to Ticarcillin/clavulanic acid, 4.2% - moderate resistance, and 20.8% - sensitivity (tab. 1).

The antibiotic susceptibility of *E. coli* isolates to the group of cephalosporins was studied, namely to Cefalotin, Cephalexin, Cefazolin (1st generation); to Cefaclor, Cefoxitin, Cefuroxime, Cefamandole (2nd generation); to Cefixim, Cefoperazone, Cefotaxim, Ceftriaxone, Ceftazi-dime (3rd generation); to Cefepim (IV generation) (tab. 1). No susceptible *E. coli* isolate was identified to Cefalotin from first-generation cephalosporins; 37.5% of isolates were moderately resistant and 62.5% of strains were found resistant. However, 70.8% and 71.4% were sensitive to Cephalexin and Cefazolin, respectively; 29.2% and 7.2% of *E. coli* isolates showed resistance; and 21.4% of *E. coli* isolates were moderately resistant to Cefazolin.

The *E. coli* isolates showed ambiguous sensitivity to the second- generation cephalosporins. 100% of *E. coli* isolates were susceptible to Cefoxitin. 25%, 83.3% and 70% of E. coli isolates exhibited

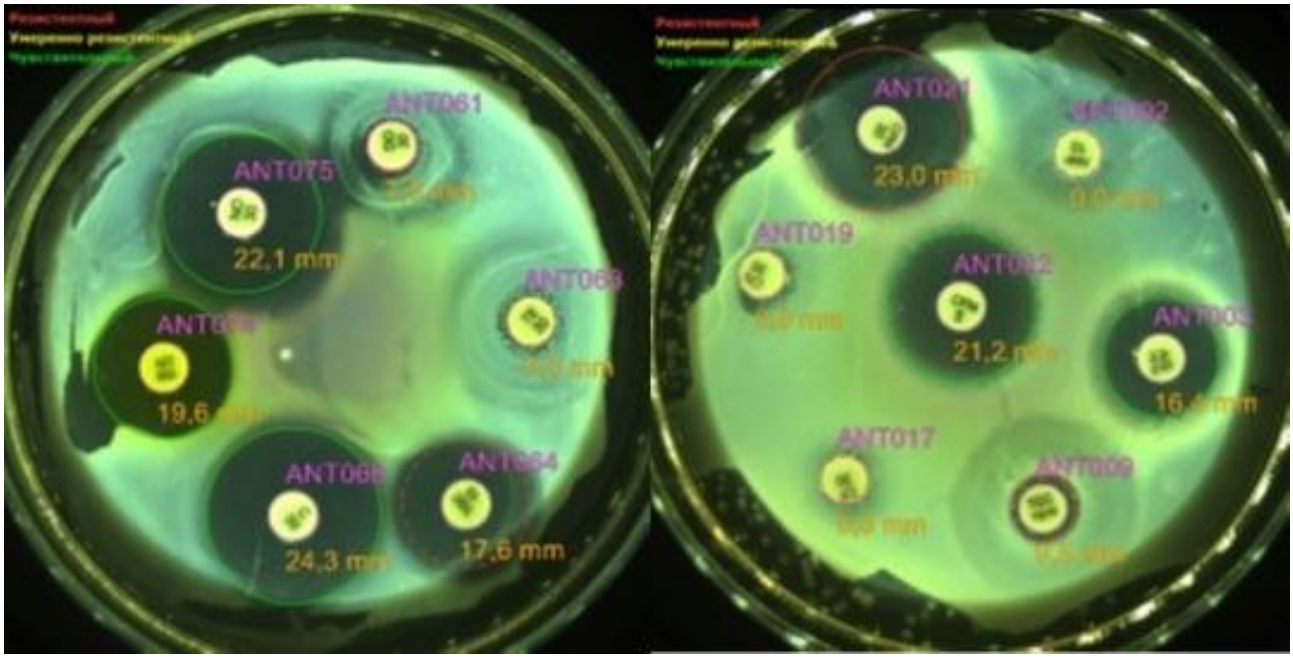

Figure 2. Antibiotic susceptibility of *E. coli* strains isolated from raw milk. ANT002 Ampicillin (r), ANT003 Ampicillin/sulbactam (s), ANT009 Ticarcillin/clavulanic acid (r), ANT017 Cefaclor (r), ANT019 Cefalexin (r), ANT021 Cefepim (r), ANT022 Cefixim (s), ANT061 Doxycycline (r), ANT063 Tetracyclinum (r), ANT064 Tigecycline (r), ANT068 Chloramphenicol (s), ANT074 Nitrofurantoin (s), ANT075 - Nitroxoline (s); «s» – sensitive; «r» – resistant; « ATU » – Area of Technical Uncertainty.

Table 1. Antimicrobial susceptibility *of E. coli* isolates (n=24).

Antibiotic	***Cultures***		
	s	***ATU***	***r***
Penicillins			
Ampicillin	-	-	100%
Ampicillin/sulbactam	95.8%	-	4.2%
Ticarcillin	35.7%	14.3%	50%
Ticarcillin / clavulanic acid	20.8%	4.2%	75%
Piperacillin	28.6%	14.3%	57.1%
Cephalosporins			
Cefalotin (I)	-	37.5%	62.5%
Cephalexin (I)	70.8%	-	29.2%
Cefazolin (I)	71.4%	21.4%	7.2%
Cefaclor (II)	25%	37.5%	37.5%
Cefoxitin (II)	100%	-	-
Cefuroxime (II)	83.3%	-	16.7%
Cefamandole (II)	70 %	10 %	20%
Cefixim (III)	87.5%	-	12.5%
Cefoperazone (III)	40%	20%	40%
Cefotaxim (III)	58.3%	16.7%	25%
Ceftriaxone (III)	83.4%	8.3%	8.3%
Ceftazidime (III)	64.3%	28.6%	7.1%
Cefepim (IV)	8.4%	20.8%	70.8%
Carbapenems			
Imipenem	20%	-	80 %
Meropenem	90%	-	10%

Note: «s» – sensitive; «r» – resistant; « ATU » – Area of Technical Uncertainty

sensitivity to Cefaclor, Cefuroxime, Cefamandole, respectively, and were resistant in 37.5%, 16.7%, 20% of isolates. 37.5% of isolates were moderately resistant to Cefaclor and 10% to Cefamandole.

The isolates showed sensitivity to Cefixim, Cefoperazone, Cefotaxim, Ceftriaxone, Ceftazidime in 87.5%, 40%, 58.3%, 83.4%, and 64.3%, respectively; resistance – in 12.5%, 40%, 25%, 8.3%, and 70.8% of cases. 8.4% strains were susceptible to Cefepim, moderately resistant – 20.8%, and resistant in 70.8% of *E. coli* isolates.

The studied isolates also showed ambiguous sensitivity to carbapenems viz. 20% of isolates were sensitive to Imipenem and 90% – to Meropenem, being resistant in 80% and 10% of strains, respectively (tab. 1).

E. coli isolates were found sensitive to Gentamicin, Kanamycin, Tobramycin, Netilin, Amikacin from the aminoglycoside group in 75%, 16.6%, 100%, 95.8% and 66.7%, respectively (tab. 2). It should be noted the high occurrence of isolates susceptible to Tobramycin (100%) and Netilin (95.8%); whereas a greater amount of isolates were resistant (41.7%) and moderately resistant (41.7%) to Kanamycin.

Table 2. Antimicrobial susceptibility of *E. coli* isolates (n=24).

Antibiotic	Cultures		
	s	m	R
Aminoglycosides			
Gentamicin (I)	75%	-	25%
Kanamycin (I)	16.6%	41.7%	41. 7%
Tobramycin (II)	100%	-	-
Netilin (II)	95.8%	-	4.2%
Amikacin (III)	66.7%	-	33.3%
Tetracyclines			
Tetracycline	16.7%	-	83.3%
Doxycycline	-	-	100%
Tigecycline	80%	-	20%

Note: «s» – sensitive; «r» – resistant; «ATU» – Area of Technical Uncertainty

83.3%, 100% and 20.0% of the investigated *E. coli* isolates were resistant to the drugs of the tetracyclines group (Tetracycline, Doxycycline and Tigecycline), respectively. 16.7% of them showed sensitivity to Tetracycline and 80% of strains to Tigecycline (tab. 2).

The investigated *E. coli* isolates were sensitive to quinolones (tab. 3).

Table 3. Antimicrobial susceptibility of *E. coli* isolates (n=24).

Antibiotic	Cultures		
	s	m	R
Quinolones			
Nalidixic acid (I)	83.33%	8.33%	8.33%
Nitroxoline (I)	100 %	-	-
Norfloxacin (II)	87.5%	-	12.5%
Ciprofloxacin (II)	83.3%	12.5%	4.2%
Ofloxacin (II)	62.5%	25%	12.5%
Lomefloxacin (II)	41.7%	45.8%	12.5%
Pefloxacin (II)	60%	-	40%
Gatifloxacin (IV)	95.8%	-	4.2%
Other antibiotics			
Nitrofurantoin	100%	-	-
Chloramphenicol	50%	-	50%

Note: «s» – sensitive; «r» – resistant; «ATU» – Area of Technical Uncertainty

100% of the tested cultures were susceptible to Nitroxoline and 83.33% to Nalidixic acid 87.5%, 83.3%, 62.5%, 41.7%, 60% of *E. coli* isolates were susceptible to fluoroquinolones of the II genera

tion, viz. Norfloxacin, Ciprofloxacin, Ofloxacin, Lomefloxacin, Pefloxacin, respectively. 95.8% of the studied *E. coli* isolates were susceptible to the fourth-generation fluoroquinolone, namely Gatifloxacin.

Sensitivity to Nitrofurantoin was proved in 100 % and to Chloramphenicol in 50% of *E. coli* isolates tested (tab. 3).

DISCUSSIONS

Therefore, the present research revealed the presence of *Escherichia coli, Staphylococcus spp*. (*Staphylococcus epidermidis, Staphylococcus aureus*) *Enterococcus spp*. isolates in all the samples of raw milk, which proved the non-compliance with the sanitary and hygienic conditions for milk production processing.

Most *Escherichia coli* isolates were found resistant to semisynthetic penicillins; 100% of the isolated cultures were resistant to Ampicillin. *Escherichia coli* isolates were selectively susceptible to inhibitor-protected penicillins, thus 95.8% of the cultures were sensitive to Ampicillin/sulbactam and 20.8% of the cultures were sensitive to Ticarcillin / clavulanic acid. *Escherichia coli* isolates were predominantly susceptible to cephalosporins.

Furthermore, the isolates showed no sensitivity dependence to the group of cephalosporins, belonging to specific generation type. At the same time, 100% of the studied isolates were susceptible to Cefoxitin and no susceptible *E. coli* isolates were detected to Cefalotin. It should also be noted the low percentage of strains sensitive to Cefepim (IV).

Moreover, there was no clear sensitivity proved to antimicrobial drugs from the carbapenem group. Most isolates showed resistance to Imipenem and were sensitive to Meropenem.

The tested *E. coli* isolates were generally susceptible to Aminoglycosides, viz. to Tobramycin (second-generation aminoglycosi-des) in 100% and to Netilin in 95.8% of isolates. At the same time, 16.6% of isolates were susceptible to Kanamycin.

The studied *E. coli* isolates were predominantly resistant to Tetracyclines and only 16.7% of the isolates were susceptible to Tetracycline. At the same time, 80% of the studied strains showed sensitivity to Tigecycline (the first-generation antibiotic of the glycylcycline group).

The tested *E. coli* isolates were also predo-minantly susceptible to quinolones and fluoroquinolones, particularly to the high activity of Nitroxoline, Nalidixic acid, and Gatifloxacin. Nitrofurantoin was also highly active against *E. coli* isolates, while only 50% of the studied *E. coli* isolates were susceptible to Chloramphenicol.

41.7% of *Staphylococcus epidermidis* isolates were resistant to Oxacillin, of which 90% were resistant to Benzylpenicillin, – 20% to Rifampicin, thus indicating an inappropriate use of antibacterial drugs in animals for disease control, prevention, and treatment.

CONCLUSIONS

1. The study results revealed the presence of *E. coli, Staphylococcus spp., and Enterococcus spp*. isolates in 100% of milk samples obtained from clinically healthy cows on livestock farms from Kiev and Poltava regions of Ukraine.
2. *Escherichia coli* isolates were predominantly resistant to semi-synthetic penicillins.
3. High percentage of *Escherichia coli* isolates were found resistant to Tetracycline (80%) and Doxycycline (100%).
4. *Escherichia coli* isolates exhibited susceptibility to Ampicillin / sulbactam, Cefoxitin (100%), Meropenem, Tobramycin (100%), Netilin, Tigecycline, Nitroxoline (100%), Gatifloxacin, and Nitrofurantoin (100%). The tested *Escherichia coli* isolates were resistant to Ampicillin (100%), Imipenem, Tetracycline, and Doxycycline (100%).
5. *Staphylococcus spp*. strains included *Staphylococcus epidermidis* (87.5%) and *coagulase-positive Staphylococcus aureus* (12.5%); 41.7% of *Staphylococcus spp*. isolates were resistant to Oxacillin, of which 90% were resistant to Benzylpenicillin and 20% to Rifampicin.

CONFLICT OF INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENT

Research was carried out with the financial support of the Ministry of Education and Science of Ukraine.

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Date of receipt of the manuscript: 08/04/2021
Date of acceptance for publication: 18/06/2021

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